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THE ROLE OF CO-OXIDATION AND COMMENSALISM IN THE BIODEGRADATION--ETC(U)
JAN 80 J J PERRY DAAG29-76-G-0159

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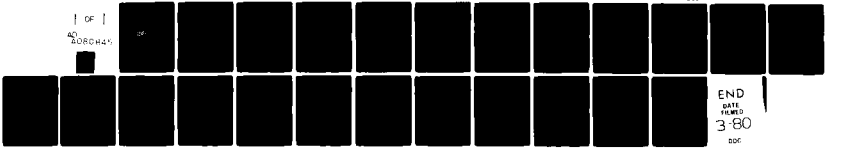

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Studies were performed on the cooxidation of various substrates, e.g. malathion, propylene, cyclohexane, polychlorinated biphenyls, and terpenes. The enzymes involved in cooxidation and commensalistic relationships whereby the products of an organism are utilized by another were investigated.

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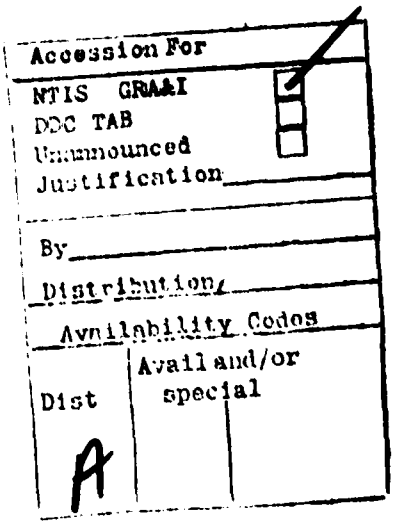


I. INTRODUCTION

This report is written to cover a number of research areas investigated during the tenure of the grant. We examined co-oxidative phenomena directly with co-substrates present and also some major enzymes responsible for the bioconversion of recalcitrant molecules in nature into molecules more amenable to biodegradation. The enzymes studied were a dioxygenase in a common soil organism, Azotobacter vinelandii, and the amine dehydrogenase system in Pseudomonas putida.

We have found that a number of commensalistic relationships exist among the thermophilic microorganisms that inhabit hot springs. One such relationship between a hydrocarbon utilizing organism and a more fastidious non-hydrocarbon utilizing organism was studied in detail. Although the thermophile work led to studies with axenic cultures not directly related to co-oxidation and commensalism, those isolated are from a significant group of organisms in nature responsible for the biodegradation of relatively recalcitrant hydrocarbon molecules. Consequently a brief outline of results with these organisms is included.

Publications are listed in the report and some research initiated during the grant period, particularly on the co-oxidation of terpenes and polychlorinated biphenyls (PCB), is not sufficiently complete that legitimate titles for future publications can be presented. It is of interest, however, that the ARO funds provided the impetus for further research on PCB's, a major environmental contaminant. We have already submitted grant requests to other agencies based on this work.

II. EXPERIMENTAL RESULTS AND DISCUSSION

a. Cooxidation and commensalism. Cooxidation can be defined as a process whereby a co-substrate is incidentally oxidized by an organism during growing on another substrates. It was originally observed in Jackson W. Foster's laboratory [E. R. Leadbetter and J. W. Foster (1960), Arch. Microbiol. 35: 92-104] and described as follows: "non-growth hydrocarbons are oxidized when present as co-substrates in a medium in which one or more different hydrocarbons are furnished for growth. Employing this principle with Pseudomonas methanica growing at the expense of methane, a series of homologous oxidation products was obtained from co-substrate gases: from ethane, ethanol, acetaldehyde and acetic acid were produced; from propane, n-propanol, propionic acid, and acetone; from n-butane, n-butanol, n-butyric acid, and 2-butanone." [J. W. Foster (1962). p. 247. In, O. Hayaishi (ed.), Oxygenases, Academic Press, New York.] Since the original observation and description of the cooxidative process, a number of studies have been conducted and those related to hydrocarbon metabolism, either with hydrocarbon as substrate or as co-substrate were the subject of a recent review [J. J. Perry (1979). Microbial cooxidations involving hydrocarbons. Microbiological Reviews 43:59-72].

In the course of studies on hydrocarbon we found, as did others, that unsubstituted cycloalkanes were not utilized by microorganisms isolated from soil. Further such organisms could not be isolated by enrichment. A typical pattern of utilization of cyclic alkanes by stock cultures is as follows:

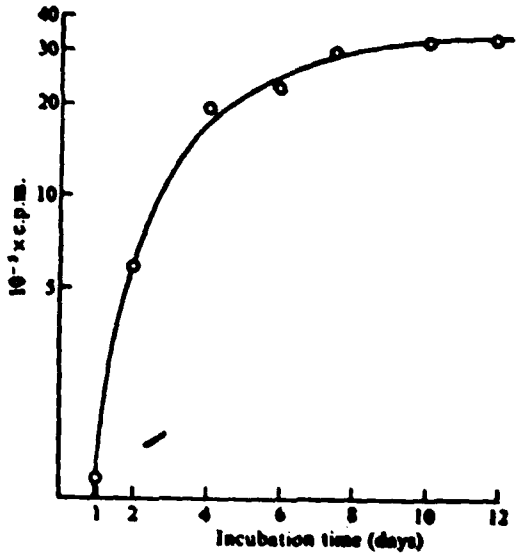
	Organism							
	M. convolutum B58	*M. vaccae* JOB5	*M. rhodochrous* 7E1C	*M. album* 7E1B1W	*N. asteroides* A116	*Strain* CY6	*M. convolutum* B22	*M. rhodochrous* OP8
Cyclopentane	–	–	–	–	–	–	–	–
Cyclohexane	–	–	–	–	–	–	–	–
Cycloheptane	–	–	–	–	–	–	–	–
Cyclooctane	–	–	–	–	–	–	–	–
Dodecylcyclohexane	+	+	+	–	+	–	+	+
Heptadecylcyclohexane	+	+	+	–	+	–	+	+
Cyclopentanone	+	–	–	–	–	–	+	–
Cyclohexanone	+	–	–	–	–	+.	+	–
Cycloheptanone	–	–	–	–	–	–	+	–
Cyclooctanone	–	–	–	–	–	–	–	–
Cyclohexanol	–	–	–	–	–	–	+	–
1,2-Cyclohexanediol	–	–	–	–	–	–	+	–
1,3-Cyclohexanediol	–	–	–	–	–	–	–	–
1,4-Cyclohexanediol	–	–	–	–	–	–	–	–
n-Tetradecane	+	+	+	+	+	–	+	+

This pattern is typical of results obtained in enrichment culture with a variety of soils and conditions. The capacity for cycloalkanone utilization is rather widespread although some organisms that grow on the cycloalkanones (CY-6) cannot utilize saturated hydrocarbons.

The mineralization of cycloalkanes by soil microflora or by mixed cultures of organisms isolated from soil was readily demonstrated. Addition of [U^{14}C]-cyclohexane to a combination of several hydrocarbon utilizing organisms with a cycloalkanone utilizing organism and n-hexadecane as co-substrate significantly enhanced the rate at which radiolabelled cyclohexane was mineralized. Typical results were:

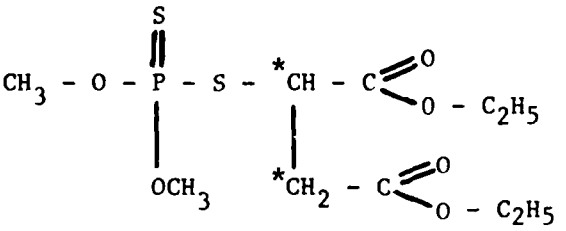
Co-substrate	$^{14}CO_2$ recovered (C.P.M.)
Glucose	73
n-hexadecane	1758
no addition	28

The addition of an inducer for the molecular oxygenase which would metabolize (cooxidize) the cyclohexane to cyclohexanone would explain the above marked effect. We also found that, although cyclohexane utilizers were not isolatable from soil, [U-^{14}C] cyclohexane was biodegraded in a rich soil as observed by the evolution of $^{14}CO_2$ from a mixture of soil + [U-^{14}C-] cyclohexane and rich marine mud:



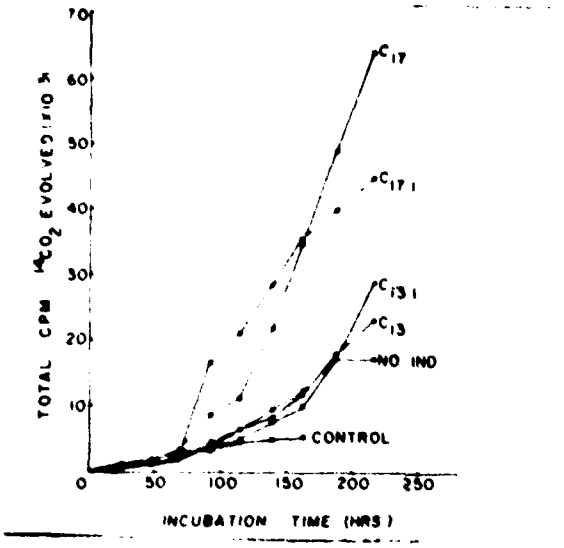
The addition of n-hexadecane or other inducers of molecular oxygenases significantly enhanced the rate of CO_2 evolution in this system. These results indicate that the biodegradation of recalcitrant molecules in nature may well be a result of two processes - (1) cooxidation of the recalcitrant molecule to a product that is more amenable to attack by other organisms, and (2) a commensalistic reaction where the product of cooxidation(s), by the concerted oxidative attack of one or more organisms, is mineralized to CO_2 and serves as growth substrate thus returning the constituent parts of the molecule to the form of living cells.

b. Biodegradation of malathion. Malathion is an effective insecticide and one of the top 10 compounds utilized for these purposes in the U.S. Studies were conducted on the effect of co-substrates on the rate at which malathion was degraded in soils from Core Creek in eastern North Carolina and in soil from a tobacco field in Wake County. Radioactive (^{14}C-carbon) pesticide was obtained and the structure with the site of labelling is as follows:

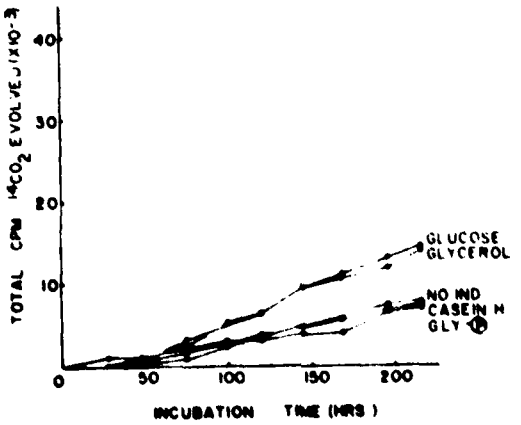


The rate of mineralization of malathion was estimated by measuring the rate that $^{14}CO_2$ was released from a mixture of soil and C^{14}-malathion in suspension. Since the label was in the 2-3 carbons of the maleate group, one can assume that total biodegradation of the molecule may well occur if these carbons are metabolized to $^{14}CO_2$. The suspension was continuously mixed and a gentle stream of CO_2-free air was passed over the surface. The effluent air was bubbled through a saturated solution of barium hydroxide to trap evolved CO_2. Co-substrates were added to the soil-malathion suspensions to determine whether any would be effective in accelerating the rate that the pesticide would be degraded. Control flasks indicated that malathion was stable under the conditions of the experimentation and traps for volatile organic compounds were not necessary.

The evolution profile of $^{14}CO_2$ from tobacco field soil samples in the presence of radiolabelled malathion and unlabelled co-substrates was as follows:



The control was soil that had been autoclaved for 50 minutes on three consecutive days. NO IND means that no co-substrate was added. The rate at which the pesticide was mineralized was significantly greater in the presence of n-heptadecane and 1-heptadecene. The addition of n-tridecane and 1-tridecene was little better than the control. Similar results were obtained with the Core Creek samples. A number of other substrates were tested in this same soil system and the results were as follows:



Glucose and glycerol were little better than the sample without added inducer. Pancreatic digest of casein (CASEIN H) and glyceraldehyde-3-phosphate (GLY-P) were not effective in increasing the rate at which the malathion was biodegraded. Other results indicate that acetate, succinate, pyruvate and citrate are not effective as co-substrates for malathion oxidation in these soils. This would suggest that a source of utilizable energy is not the limiting reaction in the biodegradation but a specific induction of enzyme(s) is a prerequisite.

These same co-substrates were tested for effectiveness in the oxidation of radiolabelled DDT, dieldrin and lindane in both the tobacco field soil and the Core Creek soil. An incubation period up to six days did not result in any release of $^{14}CO_2$ from these recalcitrant molecules. We did find, however, that lindane is biodegraded readily in other soil samples.

At the present time we cannot speculate on the pathway(s) by which malathion is degraded in the two soil types selected, but it is evident that induction of the oxidative pathway utilized in hydrocarbon oxidation is involved in some manner. The results do support the supposition that the rate at which a recalcitrant molecule will be mineralized in the environment is not solely dependent on the presence of specific organisms but also is dependent on the presence of selected compounds that can induce the synthesis of enzymes necessary for biodegradation.

c. Propylene utilization. During studies on the utilization of propane and related substrates, we noted that propylene was oxidized by most organisms capable of growth on propane but none could grow with propylene as sole source of carbon and energy. Attempts to isolate organisms by enrichment with propylene as substrate were unsuccessful but often a mixed population grew in

the enrichment composed of at least two distinctly different organisms. None of the organisms would utilize propylene after isolation in axenic culture nor could they be recombined to utilize propylene after isolation to single colonies by streaking.

After many attempts we did isolate an organism in axenic culture that would utilize propylene as sole source of carbon and energy. A selected mixed culture and the pure culture were compared for the metabolic route involved in propylene utilization. We also solved the problem of the inability of propane utilizing organisms to utilize propylene as growth substrate even though they could readily oxidize it after growth on propane.

The oxidation of various substrates in a Warburg experiment by Mycobacterium convolutum following growth on propane are:

Substrate	$Q(O_2)^*$
Propane	56
Propylene	51
1-propanol	92
Propionate	6
Acetone	148

*Microliters O_2 taken up per milligram cells per hour.

Gas chromatographic examination of the contents of the flask containing propylene were made by converting any organic acids present to the butyl ester and analyses revealed that acrylic acid was produced. The presence of acrylate was confirmed by hydrogenation of the butyl ester and rechromatographic analysis. There was a loss of the acrylate peak with a concomitant appearance of a peak corresponding to propionic acid. Addition of propylene along with propane as growth substrate for M. convolutum did not result in growth. Gas chromatographic analysis indicated that traces of acrylic acid were produced

by cooxidation and this inhibited growth of the organism. Results from other laboratories have clearly shown that acrylate is an effective inhibitor of fatty acid oxidation.

One problem with studies of propylene oxidation in mixed culture and in axenic culture is in maintaining a sufficient concentration of substrate without getting to a toxic level. An initial concentration of propylene in the enrichment flask not exceeding 1% of the atmosphere was best, and it was necessary to regas the flask after 3-4 days. The mixed culture was examined for the presence of isocitrate lyase following growth on several substrates with the following result.

Growth Substrate	Specific Activity*
Propylene	1.8
Acetate	0.7
Succinate	0
Propionate	0
Pyruvate	0

*Units per milligram of protein in which 1 unit is the amount of enzyme necessary for the cleavage of 1 micromole of isocitrate in 10 min at 30C.

These results suggest that propylene is not oxidized to the pyruvate level, by the mixed culture, before cleavage to a C_2+C_1. The C_2 would then serve as an inducer for isocitrate lyase. Trapping experiments with arsenite to block further metabolism of any pyruvate formed from [1-^{14}C] propylene (non-proliferating cell experiments) also indicated that propylene was not metabolized through pyruvate as the pyruvate formed did not contain significant amounts of radioactivity.

The indispensable role that each of the member microorganisms in the mixed culture might play is not evident at this time. This is an apparent case of cooxidation and commensalism and the organism that metabolizes the

propylene may get a growth factor from the second organism or one of the organisms may remove a toxic product of propylene oxidation. Others have reported that mixed cultures are often involved in methane oxidation and assimilation. In the best described mixed culture system for methane oxidation, one organism oxidizes methane to methanol in excessive amounts, a second is an effective scavenger of the excess methanol and a third organism produces B vitamin(s) required by the others. The interdependence of the two organisms in the mixed culture that utilizes propylene may be analogous to this system in that one alleviates toxicity of a primary oxidation product allowing both to survive.

The major difference between the mixed culture system and the axenic culture (strain PL-1) was in the way in which propionic acid was utilized. The mixed culture utilized propionate via carboxylation to methyl malonic acid, isomerization to succinate and hence through the tricarboxylic acid cycle. A determination of the isocitrate lyase levels in strain PL-1 after growth on various substrates were:

Growth Substrate	Specific Activity*
Acetate	1.3
Glucose	0
Propionate	1.0
Propylene	0.8

*Same units as above.

This is the sole organism in our extensive collection that utilizes propionic acid via a C_2+C_1 cleavage. All of the others have the methyl malonic acid pathway. The results suggest that axenic cultures that grow with propylene as sole substrate may have unusual metabolic pathways for utilization of 3-carbon compounds.

d. Thermophilic associations. A study of hydrocarbon utilization by thermophilic organisms was initiated several years ago. A number of organisms have been isolated in axenic culture and characterized from a physiological standpoint. The original work was done with soils from Core Creek, Newport River, Bogue Inlet and a receiving area for heated effluent in Allentown, Pennsylvania. Since most of the soil and water samples studied came from non-thermal environments, we decided to examine some material from hot springs.

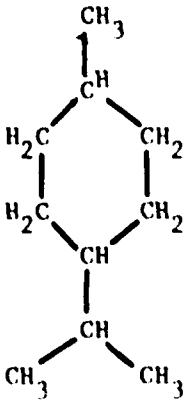
Soil and water samples were obtained from an outlet stream from a hot spring in Lower Geyser Basin, Yellowstone National Park. Enrichment cultures were prepared and incubated at 60°C for 1-2 weeks and from these several cultures were obtained. Two of these cultures, designated YS-3 and YS-4 were interesting because they had a markedly limited substrate specificity. YS-3 and YS-4 were able to grow at 60°C only with n-alkanes or 1-alkenes as substrate: dodecane through eicosane, 1-hexadecene, 1-heptadecene and 1-octadecene. No growth was observed at 60°C with alkanes from methane to undecane, alkenes from heptene through pentadecene, ketones (dodecanone through heptadecanone), acetone, alcohols nor saturated or unsaturated cyclic hydrocarbons. Neither YS-3 or YS-4 could grow at the expense of acetate, benzoic acid, pyruvic acid or any of the tricarboxylic acid intermediates. The organisms would not grow with substrates such as yeast extract, tryptone, peptone or casein hydrolysate. None of a great number of carbohydrates would support growth of YS-3 or YS-4. Addition of yeast extract to an organism growing on a substrate, e.g. n hexadecane, did not increase the rate of growth or total cells produced.

In the course of isolating YS-3 and YS-4 we observed a phenomenon that was directly related to our studies on cooxidation and commensalism. The

YS-3 was a mixed culture containing two organisms that were very difficult to separate. After extensive experimentation two axenic cultures were obtained. One, a red-pigmented organism that grew only with rich substrates such as tryptone and the other designated YS-4, a white small colony forming organism that utilized nothing except n-alkanes (see previous page) of a selected chain length. The red-pigmented organism was designated YS-8R and identified as similar to Thermus aquaticus, a thermophilic heterotrophic organism previously described.

Extensive experimentation was carried out in an attempt to ascertain the nature of the commensalistic association between YS-3 and YS8R. When YS-3 and YS-8R were combined in a medium composed of mineral salts with a single substrate, e.g., n-hexadecane, both organisms grew in a ratio of 70% YS-3 and 30% YS-8R. Insignificant growth of YS-8R grew under equivalent conditions with YS-4 as inoculum in place of YS-3. The latter organism, under axenic culture conditions will grow only on a rich medium with tryptone or the equivalent added. An effort was made to determine what product(s) of YS-3 were responsible for growth of YS-8R. A number of possibilities were tested without success. YS-3 would grow on the spent medium from YS-3 under any condition. Scanning electron microscopy did not indicate that any copious quantity of material is secreted by YS-3 nor does carbon analysis of the growth medium indicate such. The association between the two cell types examined by electron microscopy does not appear to be close. At the present time the relationship between the two is unclear but we intend to obtain more samples from the same pool and see whether the same pairing comes up on primary enrichment.

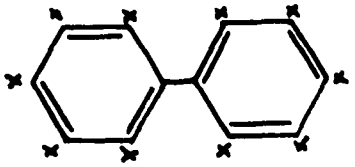
e. Cooxidation of terpenes. Terpenes are a relatively recalcitrant molecule that occur in significant quantity in the plant world. Most are derivatives of a cyclic hydrocarbon 1-methyl-4-isopropylcyclohexane as shown:



Among the cyclic terpenes are limonene in lemon, orange, caraway, dill and other oils; α-pinene, the principle component of turpentine; and camphor, a dicyclic ketone which occurs in the camphor tree.

We have initiated a program involved with cooxidation of terpenes as we have found that these cyclic hydrocarbons, as with unsubstituted cycloalkanes, are not readily utilized by microorganisms. A species of Nocardia that can utilize hydrocarbon substrates does cooxidize terpenes to menthol and several other compounds as determined by gas chromatography. We have also found that a strain of Cunninghamella elegans isolated in this laboratory and capable of growth on a wide array of hydrocarbons including crude oil will cooxidize p-menthane to menthol and other products. At the present the intermediates, other than menthol, resulting from these cooxidations are being analyzed. After determining structures we will employ enrichment culture techniques to determine which of these products of cooxidation is more amenable to biodegradation than the parent molecule.

f. Polychlorinated biphenyls (PCB). PCB's have been manufactured since 1929 and between the years 1948 and 1973 over 400,000 tons were produce in the United States. This PCB was manufactured exclusively by Monsanto Chemical Company and represents about 1/2 the world-wide production for that period. The general formula for PCB is:



x - carbon atom where chlorine or hydrogen may be substituted

There are 210 possible combinations of chlorine and hydrogen on biphenyl but about 102 actually exist assuming that there are 5 to 8 chlorines per molecule. The physical properties of PCB's, e.g. thermal stability, resistance to acid/base, inertness, have made them an important industrial chemical. They have been widely used in capacitors, as plasticizers, hydraulic fluids, adhesives, printing products, etc.

The process of manufacture and careless use of PCB's has resulted in significant amounts of these chemicals accumulating in relatively small areas. General Electric manufactured PCB containing capacitors in the upper reaches of the Hudson River for over 25 years and during this time period dumped 1,300 tons of PCB which now contaminates the Hudson River Basin. A significant part of this PCB (690 tons) is present in landfills, dumps and dredge spoils. PCB was unfortunately dumped along 211 miles of North Carolina roads by an irresponsible New York firm and amounts to an estimated 40,000 cubic yards of PCB contaminated soil. This material will probably also be placed in a landfill. These are two examples of many that could be cited where significant amounts of PCB contaminants are confined within a rather limited area.

The permanent and most satisfactory solution to such environmental contamination problems by toxic materials is total mineralization or biodegradation of the contaminant. Another major example would be the kepone waste

in the Hopewell, Virginia, area. The U.S. government often must get rid of obsolete war material, gases, etc., and the most satisfactory disposal of these would be via mineralization and under proper circumstances microbes may well be employed to accomplish this task.

We know that some man made compounds, such as DDT, have an exceedingly long half life in soil. This persistence decreases significantly under selected circumstances and in the presence of a plentiful supply of nutrient with an accompanying wide array of microbial metabolic types, as in sewer sludge, the half life of DDT is markedly shortened. I have theorized that one may augment the biodegradation of a recalcitrant molecule, present in soil, by bringing about an increase in the number of organisms present in that soil that have a metabolic capacity requisite for converting the recalcitrant compound to one more amenable to biodegradation. An example would be the increased biodegradation of cyclohexane, by a combination of organisms, when an inducer of molecular oxygenase (n-hexadecane) is added.

With the knowledge that dalapon (2,2 dichloropropionic acid) biodegradation occurs more rapidly with repeated soil application, we considered that adding this compound to soil must result either in an increased number of organisms capable of dehalogenation or the level of dehalogenases in the resident population is markedly stimulated. If so, it would follow that adding dalapon or another chlorinated compound with a rather short half life in soil, lindane (1,2,3,4,5,6 hexachlorocyclohexane), to a soil containing recalcitrant chlorinated compounds would result in a more rapid dehalogenation and consequently mineralization of the recalcitrant compound.

We obtained a series of rich soils from a number of locales and they were mixed to increase the variety of organisms present. Lindane was added to some of the mixed soil and dalapon to other samples of the soil. After

incubation at room temperature for 3-5 days, radiolabelled [UL^{14}C-]4,4' dichloro biphenyl was added to the soil and the evolution of ^{14}C-CO_2 was monitored for 21 days. A significant amount of radiolabelled CO_2 (3-4% of the total added) evolved from the soil PCB mixture when lindane was present. No measurable amount of radiolabel was released in control soils without added lindane.

The soil-PCB combination above that yielded ^{14}C-CO_2 was extracted with butanol after acidification to determine whether cooxidation products other than CO_2 were present. Thin layer chromatography of the concentrated butanol indicated that a number of metabolites were formed. This would suggest that under selected cooxidation conditions PCB may well be biodegraded. Since the 4,4'-PCB is not as recalcitrant as those containing more chlorine atoms, a considerable amount of work must be done before the process can be completely evaluated.

g. Enzymes. Many of the enzymes involved in cooxidation type reactions are inducible and present in the microbe only in the presence of a specific substance that will cause induction of the enzyme synthesis. Mixed function oxidases, dioxygenases, amine dehydrogenases, dehalogenases and many other biodegradative enzymes are rarely constitutive and as a consequence would not be effective in biodegradation in the absence of an inducer. These enzymes are generally not specific and when present will convert an array of substrates requiring only that the substrate get in close proximity to the enzyme in the microbial membrane. The constant dissolution of microbial cells in natural environments leads to the wide spread occurrence of many of these enzymes in a cell-free state but bound to soil particles. However, for a significant system of cooxidation and commensalism to operate it would require that an actively growing cell would convert substantial amounts of cosubstrate to provide nutrient for other soil microbes. We examined three

inducible enzyme systems in soil organisms and a brief description of them are as follows:

(1) Amine dehydropenase - The organism studied, Pseudomonas putida, is a common soil organism distinguished by an exceedingly broad substrate specificity and the capacity to utilize many aromatic substrates. The enzyme (amine dehydrogenase) is inducible and was isolated from the organism following growth on benzylamine. The substrate specificity of the amine dehydrogenase was independent of the amine utilized for growth. When cell-free extracts of organisms grown on benzylamine, n-propylamine or n-butylamine were subjected to polyacrylamide gel electrophoresis, a single band of enzymatic activity was detected in an equivalent position in each gel, thus indicating that an enzyme of broad specificity was involved in de..mination of these substrates. When purified to homogeneity it was found that cytochrome c or an equivalent artificial electron acceptor was required for amine dehydrogenase activity. This would suggest that the enzyme would be effective only in intact cells and would probably not be effective as a free enzyme in soil.

(2) Dioxygenase - The enzyme protocatechuate 3,4 dioxygenase, which catalyzes the conversion of protocatechuate to β-carboxyl-cis, cis-muconic acid was purified to homogeneity from the common free living nitrogen-fixing organism Azotobacter vinelandii. The enzyme had activity with a number of substrates including: pyrogallol, 3-methylcatechol and 4-methylcatechol. The substrate specificity and stability of the enzyme suggest that would be an effective enzyme in cooxidative conversions.

(3) Alcohol dehydrogenase - The alcohol dehydrogenase for secondary alcohol, e.g. cyclohexanol, was isolated from a Nocardia species. The enzyme has a specificity for secondary alcohols but without that group the enzyme displayed a broad specificity oxidizing straight chain compounds,

cycloalkanols and the cyclic diols. This enzyme would be a prime candidate for cooxidative conversions.

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IV. PERSONNEL SUPPORTED ON THIS PROJECT

William H. Underwood was a research-technician from January through June 1977. He received an M.S. degree and is now employed by Burroughs Wellcome Company in Greenville, North Carolina.

Dr. Lynne A. Stirling was a postdoctoral student from October 1, 1977, to September 30, 1979. She is now a Research Scientist at the Papanicolaou Cancer Research Institute in Miamia, Florida.